INTRODUCTION

One of the challenges in the treatment and control of *falciparum* malaria is drug resistance. The Thai–Cambodian border, in particular, is a hotspot for the emergence of antimalarial resistance: some of the earliest reports of chloroquine and pyrimethamine resistance came from this area. In addition, high failure rates of mefloquine monotherapy were documented in the 1990s.

Artemisinin combination therapy (ACT) has been promoted as an effective strategy to combat the emergence and spread of resistance. Currently, 38 countries have adopted ACT as first-line regimens for the treatment of malaria. However, a recent in vivo efficacy study on Thailand’s border with Cambodia reported an artesunate-mefloquine 28-day cure rate of 78.6%. In addition, an in vivo study on the Cambodian side of the border documented a 28-day cure rate of 85.7% in 2002. These studies suggest declining susceptibility to the artesunate-mefloquine combination in this subregion. However, it is not yet known whether these reported ACT failures are caused by true parasite resistance.

Because antimalarial resistance can emerge quickly, surveillance is a key element to a successful malaria control program. While the gold standard for monitoring drug efficacy is the in vivo trial, molecular markers could provide an inexpensive complementary tool. Before they can be used in a particular area, the relationship between specific molecular markers and in vivo resistance must be established.

One possible molecular marker is *Plasmodium falciparum* multidrug resistance gene-1 (*pfmdr1*). Single nucleotide polymorphisms (SNPs) and gene copy number have previously been associated with *in vitro* resistance to a wide array of antimalarials, including chloroquine, lumefantrine, quinine, halofantrine, mefloquine, and artesunate. However, *in vitro* and *in vivo* resistance are not always correlated. The purpose of this study was to determine whether *pfmdr1* genetic changes are associated with *in vivo* evidence of resistance to artesunate-mefloquine in Cambodia.

MATERIALS AND METHODS

Clinical study. We executed this clinical study at Pailin, Cambodia, between June and September of 2004 in conjunction with the annual monitoring of antimalarial drug efficacy in Cambodia carried out by the National Malaria Control Program. The details of this study have been previously published and were based on the WHO 2003 protocol for low transmission areas. Briefly, the eligibility criteria for this study were age >6 years, having uncomplicated *P. falciparum* malaria with no other *Plasmodium* species present, having an initial parasite density ≤100,000 asexual parasites/μL, having a measured axillary temperature ≥37.5°C, providing informed consent (by parent or guardian, when appropriate), and willing to return for follow-up. In total, 25 children and 56 adults were enrolled.

At enrollment, a brief clinical exam was performed, and a questionnaire was administered. Approximately 2 mL of venous blood was collected and frozen. Subjects were treated according to the national policy: they were given 25 mg/kg mefloquine and 12 mg/kg artesunate over 3 days (Days 0–2). The mefloquine was divided into two equal doses and given in the morning and evening on the day of enrollment (Day 0). The daily 4-mg/kg dose of artesunate was divided into two doses on Day 0 and given in one dose on the next 2 days. Subjects stayed at the clinic for 3–4 days (depending on when the parasites cleared) and had follow-up visits at 7, 14, 21, 28, 35, and 42 days after the date of enrollment. People who did not return on their own were actively sought out. At these follow-up visits, temperature was measured, and thick blood smears were used to screen for peripheral parasitemia. In instances of recrudescence of parasitemia, an additional blood sample was acquired and stored on 3M Whatman filter paper (Whatman, Middlesex, UK).

Genotyping. DNA was extracted using the Qiamp DNA mini kit (Qiagen, Hilden, Germany). Samples taken at enrollment (Day 0), Day 1, and recurrence of parasitemia were used to distinguish between recrudescence and reinfection.
The number of variants in three polymorphic genes (msp1, msp2, and glurp) was measured using size fractionization and ethidium bromide staining. The number of unique bands was determined visually and confirmed by measuring band size using Quantity One software on Gel Doc 2000 (Bio-Rad, Hercules, CA). If the recurrence specimen contained one or more of the variants seen in the enrollment or Day 1 specimen, the infection was classified as a recrudescence. If the recurrence specimen contained no variants present in the enrollment or Day 1 sample, it was classified as a reinfection.

Enrollment and recurrence samples were genotyped at pfmdr1 codons 86, 184, 1,034, and 1,042 using real-time polymerase chain reaction (PCR). A previously published protocol was followed, except that the concentration of probes was changed to 200 nmol/L.

Pfmdr1 copy number was assessed for all samples using a modification of two previous assays. The primers and a FAM-TAMRA (6-carboxylfluorescein 6-carboxy-tetramethylrhodamine) probe specific to a conserved region of pfmdr1 and the primers and a VIC-TAMRA (chemical structure not released by Applied Biosystems) probe specific to β-tubulin were multiplexed so that both genes could be assayed in the same well. The primers were synthesized by MWG Biotech (High Point, NC), and the probes were synthesized by Applied Biosystems (ABI, Foster City, CA).

PCR reactions were performed on either the ABI Prism 7000 or ABI Prism 7300. Each reaction consisted of pfmdr1 probe (150 nmol/L), pfmdr1 primers (300 nmol/L), β-tubulin probe (100 nmol/L), β-tubulin primers (100 nmol/L), Abgene QPCR Mastermix (1x), DNA (2 μL), and water up to 25 μL. If amplification of a sample was unsuccessful (no amplification, replicates being > 50% different, or a copy number < 0.6), it was repeated with 4 μL DNA. The reaction conditions consisted of 95°C for 15 minutes and then 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The cycle threshold (CT) was calculated with SDS software (ABI). DNA from strains 3D7 and Dd2 were included on each plate. The 3D7 DNA was extracted from a laboratory grown culture and Dd2 DNA was acquired from MR4 (MRA-387; ATCC, Manassas, VA).

Pfmdr1 copy number was calculated according to the following formula: copy number = \( \frac{(E_{\beta\text{-tubulin}})^{C_{T}(\beta\text{-tubulin})}}{(E_{\text{pfmdr1}})^{C_{T}(pfmdr1)}} \). The efficiency (E) of β-tubulin, which was higher than that of pfmdr1, was assumed to be 2. Pfmdr1’s efficiency, relative to that of β-tubulin, was calculated for each plate by assuming the 3D7 control has one pfmdr1 copy. Dd2, an additional control, was previously determined to have approximately four pfmdr1 copies. In our assay, Dd2 had a mean copy number of 4.27 and a coefficient of variation of 5.77%.

Statistical analysis. The clinical data were entered into Excel (Microsoft, Redman, WA) and analyzed with Stata 8.2 (StataCorp., College Station, TX). The clinical outcome of each patient was classified according to the WHO protocol. Because there were no early treatment failures, the main outcome for this analysis was the recurrence of P. falciparum parasites in the peripheral blood. Recurrences of parasitemia were further categorized as reinfections or recrudescence by the msp1, msp2, and glurp genotyping.

The copy number of samples taken before treatment and those taken at recurrence were compared using the Wilcoxon signed rank test.

The relationship between molecular changes in pfmdr1 and treatment failure was estimated using survival analysis. Because the subjects were assessed at distinct time-points, the failure time is interval-censored. Discrete Cox proportional hazards model was used to take this censoring in account. This model was estimated using regression with a complementary log-log link. Indicator variables representing four time periods (0–21, 22–28, 29–35, and 36–42 days) were included, whereas a constant term was not estimated. All variables were assessed for the proportional hazard assumption. The main outcome was time to recrudescence. Subjects who were lost to follow-up were censored on their last visit. Subjects who developed a P. vivax infection or a P. falciparum reinfection were censored on the day of that second diagnosis. The precision of all hazard ratio estimates were evaluated by calculating the confidence limits ratio (CLR).

The genotype at codons 86, 184, 1,034, and 1,042 in pfmdr1 were combined into haplotypes, as previously described. The comparison between haplotypes I and III was made because these haplotypes had the largest sample size and because a previous in vitro study in Cambodia found haplotype III was associated with mefloquine resistance and haplotype I with mefloquine sensitivity. Samples that were a mixture of haplotypes I and III were coded as haplotype III. The relationship between copy number and time to recrudescence was not linear on the log hazard scale. Therefore, copy number was coded as a binary variable of less than three copies and at least three copies to reflect the relationship between copy number and time to recrudescence and to meet the proportional hazards assumption of the model.

The following covariates were evaluated as potential effect measure modifiers and confounders: previous use of antimalarials, initial parasite density, age, sex, and hematocrit. Initial parasite density was coded as a binary variable, with the cutoff point of 80,000 parasites/μL. Effect measure modification was assessed with the Wald test for the interaction term in the model (P ≤ 0.1 was considered significant). All non-effect measure modifiers were evaluated in a directed acyclic diagram to select a sufficient set of covariates to control for confounding.

The population attributable rate fraction (AFp) and 95% confidence intervals (CIs) were calculated. The specific formula for AFp was \( \frac{(HR_{adj} - 1) \times p}{HR_{adj}} \) where HRadj = adjusted hazard ratio, and p = proportion of cases that were exposed.

The relationship between molecular change in pfmdr1 and parasite clearance time was assessed using discrete Cox proportional hazards model in the same manner as described above.

Ethics. Informed consent was obtained from all adult subjects and from the parent or guardian of all minors. The pfmdr1 genotyping and data analysis were approved by the Institutional Review Boards at the United States Naval Medical Research Unit No. 2 and at UNC Chapel Hill School of Public Health. Both the genotyping and the efficacy study was approved by the National Ethics Committee for Health Research, Ministry of Health, Cambodia.

RESULTS

Efficacy study. Of the 81 people enrolled, 10 were lost to follow-up, 7 were diagnosed with P. vivax, and 25 people...
experienced late treatment failure between Days 14 and 42 (Figure 1). *msp1, msp2,* and *glurp* genotyping determined that 13 of the 25 recurrences were caused by recrudescence of the original infection. In the enrollment samples, there were two *msp1* variants, five *msp2* variants, and five *glurp* variants. The prevalence of the most common *msp1/msp2/glurp* haplotype was 8%, which implies there is a low probability of a reinfection having an identical haplotype to the original infection.

Enrollment samples were available for *pfmdr1* genotyping for all but one subject. This subject was a child who recrudesced on Day 14. Clinical and demographic information for the remaining 80 subjects are presented in Table 1.

**Genotyping of enrollment samples.** Of the 80 enrollment samples, 75 (93.8%) were successfully genotyped at all four *pfmdr1* codons. Of the remaining five samples, genotyping of one sample was unsuccessful at *pfmdr1*–86, one was unsuccessful at *pfmdr1*–1034, and three were unsuccessful at *pfmdr1*–1042. Most samples had either the I or III haplotype (Table 2). *pfmdr1*–86-Tyr was not observed.

*pfmdr1* copy number was successfully determined for all enrollment samples. The median copy number was 1.50 (range, 0.60–6.28), and 18.8% contained three or more copies. Elevated copy number was only observed in the presence of 86-Asn, 1034-Ser, and 1042-Asn (haplotypes I and III). There was no association between copy number and *pfmdr1*-184 genotype (ANOVA, F = 0.51, P = 0.479).

**Comparison of enrollment and recurrence *pfmdr1* genotypes.** *pfmdr1* copy number was determined for all recrudescent (N = 12) and all reinfection (N = 12) samples; 75.0% (9/12) of the reinfection samples and 66.7% (8/12) of the recrudescent samples had a higher copy number than the corresponding enrollment sample. These differences were not statistically significant (Wilcoxon signed rank test, for recrudescence: z = 1.26, P = 0.209; for reinfection: z = 1.57, P = 0.117). However, when comparing all enrollment and recurrent samples, the difference was statistically significant (Wilcoxon signed rank test, z = 2.06, P = 0.040). Therefore, artemisinine-mefloquine treatment seems to select for increased *pfmdr1* copy number.

Four recrudescent and 10 reinfection samples were genotyped for *pfmdr1* polymorphisms. When comparing the haplotypes at enrollment and recurrence for the reinfection samples, five were identical, one sample changed from haplotype IV to III, two samples changed from being mixed (haplotypes I and III) to haplotype III, and two samples changed from having haplotype III to being mixed (haplotypes I and III). The four recrudescent samples had identical haplotypes to the enrollment sample (three of these paired samples had haplotype III and one had haplotype I). Thus, there seems to be no selection of a particular *pfmdr1* haplotype because of artemisinine-mefloquine treatment.

**pfmdr1 and time to recrudescence.** Subjects with increased *pfmdr1* copy number recrudesced sooner and more often than subjects with low copy number (Figure 2; Table 3). The crude hazard ratio for increased copy number (at least three copies) on time to recrudescence was 8.30 (95% CI: 2.60–26.43). Adjusting for confounders resulted in a hazard ratio of 7.91 (95% CI: 2.38–26.29). These results were not dependent on the statistical model, because the hazard ratio from a continuous Cox proportional model was similar in size and precision. Thus, *pfmdr1* copy number is strongly related to time to recrudescence.

For *pfmdr1* haplotype, 11 of 12 recrudescences occurred in subjects infected with parasites with haplotypes I and III. One subject with parasites with an incomplete haplotype also recrudesced. None of the four subjects with parasites with haplotype IV recrudesced; however, the small sample size prevents further evaluation of this haplotype. The crude hazard ratio of time to recrudescence comparing haplotype III to I was 2.22 (95% CI: 0.28–17.34). Adjusting for confounders resulted in a slightly lower hazard ratio (1.88; 95% CI: 0.24–15.00). There seems to be little difference between haplotype III and I on time to recrudescence; however, the estimate is very imprecise (CLR = 61.36).

To investigate the impact of increased *pfmdr1* copies on recrudescence in the entire cohort, the population attribut-

![Figure 1](flowchart.jpg)

**Figure 1.** Flowchart describing the results of the *in vivo* efficacy trial in 2004 in Pailin, Cambodia.

### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
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<tr>
<td>Men (%)</td>
<td>57 (71.3%)</td>
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<tr>
<td>Median age (range)</td>
<td>20 (6–65)</td>
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<tr>
<td>Geometric mean parasite density [parasites/μL (SD)]</td>
<td>$1.89 \times 10^9$ ($1.16 \times 10^9$)</td>
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<tr>
<td>Mean hematocrit (SD)</td>
<td>38.7 (5.3)</td>
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<td>Previous use of antimalarials* (%)</td>
<td>28 (37.8%)</td>
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</table>

* In the past month by self-report (n = 74).

### Table 2

*pfmdr1* haplotypes of samples taken at enrollment with successful genotyping at all codons

<table>
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<tr>
<td>I</td>
<td>13</td>
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<tr>
<td>III</td>
<td>57‡</td>
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<tr>
<td>IV</td>
<td>4‡</td>
</tr>
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<td>V</td>
<td>1</td>
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</table>

*Amino acids representing the mutant genotype are in bold.
* From reference 11.
† Five samples were mixed at 184.
‡ One sample was mixed at 1,042.
able fraction was calculated. Using the prevalence of greater than three pfmdr1 copies in the cases (58.3%) and the adjusted hazard ratio (7.91), the population attributable fraction of recrudescence for increased pfmdr1 copy number was 50.9% (95% CI: 4.1–74.9%). pfmdr1 copy number seems to contribute to the cause of one half the cases of recrudescence observed in this study.

When the outcome of all recurrences of parasites (recrudescence + reinfection) was used, the estimate of effect for copy number was lower (unadjusted HR = 4.51, 95% CI: 1.99–10.23; adjusted HR = 4.02, 95% CI: 1.73–9.34). In contrast, the hazard ratio for time to recrudescence for haplotype III was higher but very imprecise (unadjusted HR = 4.78, 95% CI: 0.64–35.64; adjusted HR = 4.40, 95% CI: 0.59–33.02). Therefore, the estimated effect of pfmdr1 haplotype but not copy number changes depending on whether the treatment failures were PCR corrected. This suggests that if some misclassification of recrudescence and reinfection had occurred in this study, the interpretation of the effects would not be substantially different.

The pfmdr1 copy number is a potential screening tool for drug resistance. Having parasites with greater than three pfmdr1 copies before treatment identified cases of recrudescence with a sensitivity and specificity of 58.3% and 88.2%, respectively, and a positive predictive value of 66.7%. Including information on either pfmdr1 haplotype or initial parasite density increased the specificity but decreased the sensitivity of predicting recrudescence (at least three copies + haplotype III: sensitivity = 50.0%, specificity = 91.2%; at least three copies + parasitemia ≥ 80,000: sensitivity = 50.0%, specificity = 91.2%).

**pfmdr1 and parasite clearance time.** Of the 80 subjects, 4 (5.0%) cleared the parasitemia by Day 1, 41 (51.3%) cleared by Day 2, 27 (33.8%) cleared by Day 3, and 8 (10.0%) cleared by Day 4. The parasite clearance times observed in this study are longer than reported previously for artesunate-mefloquine.28

Increased copy number was not associated with delayed parasite clearance when controlling for parasite density and hematocrit (Table 4). There was a slight association between pfmdr1 haplotype and delayed parasite clearance time, although the estimate was imprecise.

**DISCUSSION**

In this study, pfmdr1 copy number at the time of treatment initiation was strongly associated with recrudescence after

<table>
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<th>Level</th>
<th>No. subjects</th>
<th>No. recrudesced</th>
<th>Crude hazard ratio</th>
<th>Adjusted† hazard ratio</th>
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<td></td>
<td></td>
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<td>Copy number</td>
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<tr>
<td>&lt; 3</td>
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<td>8.30</td>
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<td>22</td>
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<td>3.09</td>
<td>1.00–9.61</td>
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* Confidence limit ratio = upper limit/lower limit.
† Adjusted for initial parasite density and hematocrit.
‡ Referent level.
The main limitation of copy number on recrudescence genotype had minimal variation, with some sensitive selection for mefloquine resistance and artesunate-mefloquine copy number in parasitaemia. Also, parasite clearance is predominantly caused by artesunate, and no effect of pfmdr1 copy number on parasite clearance time was observed in this study. Therefore, the effect pfmdr1 copy number on recrudescence is most likely caused by the effect of pfmdr1 on mefloquine sensitivity and not artesunate sensitivity.

We observed an increase in pfmdr1 copy number in parasites isolated after artesunate-mefloquine treatment. Because mefloquine has a much longer half-life than artesunate (14–41 days versus 1 hour), resistant parasites were probably selected by exposure to subtherapeutic concentrations of the single drug mefloquine. Selection for mefloquine resistance in Pailin may have also been facilitated by the pre-existence of resistance to this drug. The observation suggests that ACT selects for resistance to the non-artemisinin partner drug.

Strengths of this study include the fact that genotyping was done directly on peripheral blood samples, which negates the potential bias caused by culturing. Another strength is its 42-day follow-up with PCR correction. The main limitation of this study is the small sample size, which affected the power and the precision of the effect estimates, especially for pfmdr1 haplotypes. Another limitation is that not all predictors of recrudescence were measured, such as pharmacokinetics, host genetics, immunity, and nutritional status.

Molecular markers of drug-resistant malaria, such as pfmdr1, are promising tools for the surveillance of drug resistance. pfmdr1 copy number is not only strongly associated with recrudescence to artesunate-mefloquine but also with Coartem failure (artemether-lumefantrine). Surveillance for increased pfmdr1 copy number could aid malaria control efforts by pinpointing areas where these drugs may be failing.

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### TABLE 4

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* Confidence limit ratio (CLR) = upper limit/lower limit.
† Adjusted for initial parasite density and hematocrit.
‡ Referent level.
References


